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14. ABSTRACT

The long-term objective of this research is to develop a device for treating spinal cord injury. The specific aims of the proposed study are to test new types of collagen tubes and porous collagen scaffolds. Moreover we will be investigating the effects of incorporating genes from nerve growth factors into the collagen scaffolds and seeding the scaffolds with marrow-derived mesenchymal stem cells. The standardized defect site is a 5-mm gap in the rat thoracic spinal cord. Our principal method of evaluation is histomorphometry. During the past project year the following were accomplished, toward achieving the objectives of determining the effects of selected design variables on the reparative processes in spinal cord defects: 1) produce oriented pores in the porous collagen scaffold, 2) non-virally transfect undifferentiated marrow-derived mesenchymal stem cells (MSCs) in monolayer, with plasmid DNA encoding a neurotrophic factor, 3) supplement the collagen scaffold with plasmid DNA, and 4) transfect MSCs grown in the gene-supplemented collagen scaffold, with plasmid DNA for a neurotrophic factor.

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I. INTRODUCTION

The long-term objective of this research is to develop a device for treating spinal cord injury. The specific aims of the proposed study are to test new types of collagen tubes and porous collagen scaffolds. Moreover we will be investigating the effects of incorporating genes from nerve growth factors into the collagen scaffolds and seeding the scaffolds with marrow-derived mesenchymal stem cells. The standardized defect site is a 5-mm gap in the rat thoracic spinal cord. Our principal method of evaluation is histomorphometry.

Our supposition is that an appropriate synthetic substrate (*i.e.*, the collagen scaffold) will mimic or perhaps improve upon the documented ability of peripheral nerve grafts to promote the regrowth of injured spinal axons, and that the seeded stem cells will differentiate under the influence of the endogenous regulators to a support cell phenotype. Collagen-based materials are the bio-inspired biomaterials being used for the fabrication of the tube, covering film and porous scaffold.

II. BODY

During the past project year, research focused on the following technical objectives:

1) produce oriented pores in the porous collagen scaffold,
2) non-viral transfection of undifferentiated marrow-derived mesenchymal stem cells (MSCs) in monolayer, with plasmid DNA encoding a neurotrophic factor,
3) supplement the collagen scaffold with plasmid DNA, and
4) transfect MSCs grown in the gene-supplemented porous collagen scaffold, with plasmid DNA encoding a neurotrophic factor.

A. Produce Oriented Pores in the Porous Collagen Scaffold

1. Background

The pore characteristics of absorbable, sponge-like tissue engineering scaffolds, which generally govern their performance, include the void fraction (percent porosity), pore diameter distribution, and pore interconnectivity. For applications in which a scaffold is to bridge gaps in tissues comprising uniaxially aligned cells (*viz*., spinal cord) , the orientation of the pores of the scaffold may also play a critical role in its performance. The advantages of using a scaffold with axially oriented pores alone or within an entubulation strategy have been well documented in the literature, for both the peripheral and central nervous systems. The objective of the present study was to implement a straightforward technique, based on uniaxial freezing and subsequent freeze-drying, for the production of collagen-based scaffolds with axially oriented pores, to be used as nerve guidance matrices for defects in the spinal cord.

2. Materials and Methods

Collagen scaffolds were produced according to the following procedure. A suspension of porcine type I/III collagen (Geistlich Biomaterials, Wolhusen, Switzerland) in dilute hydrochloric acid (pH=3.2) was prepared with either 0.5% w/v or 2% w/v collagen. The slurry was degassed via centrifugation and injected into cylindrical holes, 3 mm in diameter, machined into a polyethylene plate, 15 mm thick. Prior to injection of the collagen suspension into the polyethylene mold, copper caps were fitted into the bottom openings of the holes in the mold. Subsequently, the copper-fitted end of the mold was rapidly cooled at two different freezing temperatures, yielding four experimental groups (Table 1). Groups I and II were frozen by directly placing the mold onto the shelf of a freeze-dryer (VirTis, Gardiner, NY, USA) pre-

cooled to -40°C, for one hour; for Groups III and IV, the freezing of the suspension was achieved by placing the mold onto a copper plate, previously cooled with liquid nitrogen (LN_2), for 10 minutes. The copper plate onto which the mold was placed was pre-cooled by pouring LN_2 on it, until a film of LN_2 formed on its surface; during the 10 minutes necessary for the freezing of the entire length of the scaffolds (15 mm), LN_2 was continuously supplied to avoid temperature changes of the plate. After freezing had occurred, the molds were immediately transferred to a freeze dryer for subsequent lyophilization.

The scaffolds were freeze-dried overnight, then removed from the mold and cross-linked by dehydrothermal treatment (DHT) at 105 °C and 30 mmHg for 24 hours. This treatment leads to the formation of covalent linkages among the polypeptide chains of the collagen fibers without denaturing the collagen into gelatin.

In view of a future implantation of the matrices into a 5-mm long defect in the adult rat spinal cord, the cross-linked scaffolds were cut into 5-mm long cylinders using a razor blade. One 5-mm long sample was obtained from each mold. The cylindrical scaffolds received two transverse cuts, at distances of 3 mm and 8 mm from the bottom, respectively. Both ends of the scaffolds resulting from the cuts were discarded.

Qualitative examination of the pore structure was carried out by means of environmental scanning electron microscopy (ESEM). Transverse sections through the middle of the 5-mm long scaffolds were analysed by means of optical microscopy to determine the mean pore diameter and the aspect ratio of the pores. Longitudinal sections through the upper 2.5 mm length of the scaffolds were detected for the evaluation of the mean pore orientation. A total of 6 samples for each experimental group were processed and analyzed as described in the following. Specimens allocated for light microscopy were embedded in glycol methacrylate. The embedded specimens were then sectioned at 5 μm thickness with a microtome, and mounted onto standard glass microscope slides. Before being cover-slipped, the sections were stained with aniline blue in order to enhance the contrast between the background and the collagen fibers under the optical microscope.

Images of the sections were taken at low (4X objective lens) and high magnification (20X) using an Olympus BX51 light microscope (Olympus Optical Co., Tokyo, Japan). The low magnification micrographs were captured to provide a wider view of the porous structure, thus allowing an estimation of the homogeneity and distribution of the pores throughout the entire section. For both the transverse and longitudinal sections, two different areas were then analyzed from high magnification pictures: 1) the peripheral area was assumed as the outer 400 μm of the scaffold diameter; and 2) the central area was defined as the area covering the central 1.2 mm of the scaffold diameter. Since the central area was much larger than the peripheral one, the mean pore diameter and pore orientation of the scaffold are here defined as the pore diameter and the pore orientation calculated from the analysis of the central zone. Fig. 1 shows the number and the location of the high magnification pictures captured for the analysis of the transverse and longitudinal sections respectively.

A total of at least 100 pores for each region were considered for the evaluation of the various pore parameters. The linear intercept method, often employed for the evaluation of the mean pore diameter of porous materials, was applied here to determine the mean diameter, pore aspect ratio, and the orientation of pores, using Scion Image Analysis 4.0.3 software (Scion Corporation, Frederick, MD).

A linear intercept macro written for the Scion Image software involves the construction of an ellipse that best fits the mean cross-section of a pore, for each image analysed. When

analyzing the micrographs of longitudinal sections of the scaffolds, the angle of orientation of the best-fit ellipse, obtained from the linear intercept analysis, was calculated and assumed to be representative of the mean pore orientation in each image. The orientation of the pores was expressed in terms of an orientation index, *OI*. OI is equal to -1 in case of radial orientation and equal to 1 in case of axial orientation. The pore volume fraction of the scaffold and specific surface area were also determined.

3. Results

The mean pore diameter in cross-sections through the cylindrical scaffolds ranged from about 20 to 60 μm and varied with the collagen concentration in suspension and the temperature at which the suspension was frozen (Table 2). There was an inverse relationship between pore diameter and collagen concentration, and smaller pore diameters yielded by the samples frozen in liquid nitrogen (Table 2). Two-factor ANOVA demonstrated that both of these independent variables significantly affected the mean pore diameter of the central area of the scaffold cross-section ($p<0.0001$; power=1). Preliminary ESEM micrographs demonstrated a variation of pore characteristics along the full length (15 mm) of the original scaffolds. However, the pore diameter and orientation did not appear to change noticeably in the lower half of the scaffolds, along the 5-mm length chosen to be representative for the matrices studied here. Cross-sections corresponding to the middle cross-sections of the 5-mm matrices were here assumed representative of the mean pore size of the scaffold and analyzed.

Larger values of pore diameter were observed in the peripheral zone of the scaffold, when compared to the central zone, for Groups I and III (Table 2). The results for Groups II and IV (Table 2) were not considered reliable, because the high deformability of the samples, in spite of the cross-linking treatment, caused their compression during the processing (scaffold embedding and cutting), thus compromising the pore structure. Moreover, when comparing peripheral and central zones, it is worth noting that the peripheral zone displayed larger coefficients of variation, indicating a wider distribution of pore diameters (Table 2).

The aspect ratio of the pores, related to the pore morphology, was not significantly affected by either the collagen concentration or the freezing temperature (Table 2), and was close to 1 for all the experimental groups, suggesting that the uniaxial freezing of the collagen slurry described here resulted in the formation of nearly circular pores. This morphological result was also confirmed by qualitative analysis of ESEM cross-sectional micrographs.

Light micrographs of longitudinal sections through the upper half of the 5-mm long scaffolds were analyzed to determine the mean pore orientation (Table 3), as described previously for the calculation of *OI* and the angle of orientation. The mean pore orientations of the four experimental groups were not statistically different, indicating that the concentration of collagen in suspension and the freezing temperature did not significantly affect the direction of growth of ice crystals during the freezing of the collagen slurry (2-factor ANOVA for collagen concentration and temperature, $p=0.40$ and $p=0.54$ respectively). The orientation index, *OI*, in the central area ranged from about 0.8 to 0.9, indicating a prevalently axial pore orientation, *i.e.*, a prevalently axial freezing process. There were high coefficients of variation for *OI* in Groups II and III, and non-normal distribution of the data for *OI* in Group III (Table 3).

The pore channels in the peripheral zone seem to be more radially oriented if compared to the pores found in the central zone. As found for the central pore orientation, the collagen concentration and the freezing temperature do not affect significantly the orientation of the pore channels in the peripheral area ($p=0.08$ and $p=0.37$ respectively).

The pore volume fraction was a function of only the collagen concentration, and was 98.4% for Groups I and III (2% w/v collagen) and 99.5% for Groups II and IV (0.5% w/v collagen), as reported in Table 4.

The values of the specific surface area are also listed in Table 4 for each different mean pore size obtained for the four experimental groups. As mentioned above, the mean pore size measured from the analysis of the central area of the scaffold (inner 1.2 mm) was assumed here as mean pore size of the matrices. The Group III scaffolds with the smallest pore diameter correspondingly (20 μm), had the highest specific surface area (SAV).

A qualitative ESEM analysis on transverse and longitudinal sections has been performed to detect the morphology of the pore structure and its homogeneity throughout the scaffold. A preliminary study was focused on the analysis of the pore characteristics along the full length (15 mm) of the cylindrical matrices. Longitudinal pore channels could be observed throughout the samples, and increasing pore diameters were detected going from the bottom to the top of the scaffolds).

4. Summary

We have developed a simple method for producing uniaxially oriented pores in the collagen scaffolds.

B. Non-viral Transfection of Undifferentiated Marrow-Derived Mesenchymal Stem Cells (MSCs) in Monolayer, with Plasmid DNA Encoding a Neurotrophic Factor

1. Background

The reparative process in the spinal cord will likely need to include agents of regeneration including; neurotrophic factors. The infusion of therapeutic molecules has limitations in spinal cord repair. These molecules, if delivered systemically, can undergo metabolic degradation by the liver. Even if delivered locally the molecules have a short half-life, which would require a continuous administration of the drug. Finally, there are some difficulties in targeting a specific cell population such as neurons. A number of vectors have been developed to introduce genes encoding therapeutics into defects n tissue in order to prolong a high level and localized delivery of specific neurotrophic factors. We have chosen non-viral gene transfer methods because of their safety relative to viral vectors.

Glial cell-derived neurotrophic factor (GDNF) was selected for investigation because it has shown potent actions on damaged and diseased neurons. GDNF has been reported to: be able to reverse some aspects of aging in monkeys, have significant effects on the symptoms of Parkinson disease when directly infused in the monkey brain, and prevent nigral cell death when recombinant human GDNF is injected either before or after lesions produced in the rat substantia nigra. Finally, GDNF is expressed in many tissues and by many cell types throughout the body, including neurons. The plasmid for GDNF (pGDNF) which contains the Green Fluorescent Protein (GFP) gene under the control of the Internal Ribosome Entry Site (IRES) immediately early promoter, was a gift from Professor M. Bohn (Northeastern University, Chicago).

2. Materials and Methods

Rat MSCs were isolated from both the femur and the tibia of young rats (<6 weeks old). Cells from only one animal were used to eliminate variability related to inter-animal differences. Isolated MSCs were then plated in 150 cm^2 flasks at 5000 cells/cm^2 and expanded in the expansion media prepared with low glucose Dulbecco's modified Eagle's medium (DMEM)

(GIBCO, Cat#11885-084), 20% of FBS and 1% of antibiotics – penicillin/Streptomycin. Once the cells in P0 reached about 90% confluence, they were detached, resuspended and replated to P1 before being frozen at -80°C. One day prior to the transfection, the cells were counted by hematocymetry and plated at 8000 cells/cm^2 in well-plates.

The plasmid DNA was amplified in competent Escherichia coli (DH5 from Invitrogen) and isolated from the bacteria with the Mega QIAfilterTM Plasmid kit (Qiagen). When measured to assess the purity of plasmid DNA obtained, the ratio of absorbance at wavelengths of 260 and 280nm ranged around 1.75 and 1.8. The integrity of the amplified plasmid was determined by agarose gel electrophoresis; the isolated pGDNF was compared uncut, and cut with Not I and Xho I, to the original plasmid. The size of pGDNF was 6.75kb.

Two lipid transfection agents were investigated to enhance transfection of the MSCs in monolayer with pGDNF: GenePorter2 (GP2) and Lipofectamine 2000 (L2000).

GenePorter2 (GP2) For the GP2 experiment, 0.2, 0.5, and 2µg pGDNF was loaded in the plasmid/lipid complex, where a ratio between the plasmid DNA and the liposome (µg:µl) was fixed at 5 (n=6). Plasmid DNA was transfected at 1 week after cell seeding at about 70% confluence using GenePorter 2 according to a standard protocol. Two control group received 2 µg of the naked plasmid or no plasmid (n=6).

Lipofectamine 2000 (L2000) In the L2000 experiment, 1.6 µg was employed at a ratio of 1.6 µg plasmid to 4 µl L2000 (n=6). In order to compare the two systems, the cumulated release of GDNF in the medium was also normalized to the amount of pGDNF initially incorporated in the lipoplexes. Plasmid DNA was transfected at 9 days after cell seeding at about 95% confluence using Lipofectamine 2000 CD Reagent (Invitrogen, Cat# 12566-014). Two different transfection media were compared: Opti-Pro (GIBCO, Cat# 12309) with 4mM L-Glutamine and DMEM low glucose (GIBCO, Cat#11885-084). The mixture complex was added directly to the cells in serum-free media (DMEM low glucose) and incubated at 37°C for 24 hours.

Control MSC monolayer cultures received 1.6 µg of the naked plasmid or no plasmid (n=6).

Micrographs of MSCs expressing GFP were taken in a fluorescence microscope, with every change of the media. Determination of transfection efficiency was performed after 24 hours and on days 3, 6, 9, 12, 14 post-transfection by fluorescence microscopy.

The media were collected (0.3ml) on days 3, 6, 9, 12, and 14 post-transfection for ELISA analysis (Duo-Kit ELISA for the human GDNF protein; R&D Systems) and stored at -20°C until the ELISA was performed. Chemiluminescent ELISA assays (R&D Systems) were run in a multilabel Victor 3 microplate counter and analyzed using Wallac 1420 software (Perkin Elmer Life Sciences). The amount of GDNF released in the medium was monitored by reading absorbance at 450nm minus absorbance at 540nm to correct for optical aberrations.

Cells were obtained at the day of the transfection and at the end of the experiment (14 days) to evaluate for cell number. Briefly, the medium was removed from the culture, the cells were washed with phosphate-buffered saline (PBS), and then the cells were detached by incubation with collagenase followed by incubation with 0.25% trypsin/EDTA. Rat MSCs were recovered by centrifugation and resuspended in PBS. The number of cells was determined following the DNA quantification of the samples with a Pico-Green Assay (Appendix F).

3. Results

In the first experiment GDNF expression was observed for all monolayers transfected with a cationic reagent (Fig. 5). Control MSC monolayers (n=6 per condition), cultured without the addition of the plasmid and with the naked plasmid, displayed small amounts (< 1 ng/ml) of GDNF released into the medium at the collection time points (Fig. 5). The results of the accumulated release of GDNF (Fig. 6) showed some noticeable differences in the expression profile of GDNF among the transfection reagents. During the first 12 days, the production of GDNF was linear ($R^2 \approx 0.99$) and reached about 1.2 ng/ml per day in the GP2 study (2 μg plasmid load), whereas the use of L2000 (1.6 μg plasmid load) gave about 0.2 ng/ml per day for both media.

When considering the total accumulated and normalized GDNF released at the end of the two-week period, the result with GP2 was about 3-fold higher than L2000 (Fig. 6). Two-factor ANOVA showed a significant effect of both time ($P<0.0001$; power=1) and transfection reagent/medium ($P<0.0001$; power=1) on the cumulative GDNF release. Two-factor ANOVA was also performed to determine the effects of medium (for the L2000 data) and time on the accumulated GDNF release. The statistical analysis showed a significant effect of the transfection medium ($P<0.0001$; power=1) and a moderate effect of the time ($P<0.008$; power=0.88) on GDNF production.

A PicoGreen Assay was performed for each condition (n=6) at Day 14, and the DNA content was compared to a control (n=6) where MSCs were grown in the same medium conditions, but not transfected. The DNA content for the GP2 experiment was 460ng/well whereas its control was 530ng/well. In static conditions, there were 13% fewer cells after transfection than for the control. The DNA content for the L2000 experiment was 130ng/well for both media whereas the control was 520ng/well in OptiPro, and 250ng/well in DMEM; these results showed respectively 75% and 50% fewer cells for the transfected cells than the control.

This experiment demonstrated the importance of the selection of the transfection reagent and the expansion medium, which affect the cells morphology and density.

In the second experiment, production of GDNF could be noted for loading of plasmid DNA as low as 0.2μg per well. The ELISA assay also showed that the expression of pGDNF decreased with time for any initial load contained in the lipoplexes. The maximal transfection was obtained with the highest initial dose of plasmid reaching more than 8ng/ml at Day 3 and maintaining the highest levels of expression throughout the 2-week period of the experiment.

The accumulated release of GDNF for an initial 2 μg load of pGDNF showed a production of 22 ng/ml after a 2-week period. Two-factor ANOVA of the cumulative release of GDNF demonstrated significant effects of both time and plasmid amount on the release of GDNF ($P<0.0001$; power=1).

4. Summary

The results demonstrated the possibility of using non-viral transfection conditions *in vitro* to enable MSCs to express a selected neurotrophic factor, GDNF, in therapeutic doses. It has been shown in previous studies that GDNF, at 1 and 100 ng/ml, significantly increased by nearly 100% the number of trigeminal ganglion sensory neurons in culture at 5 days post-plating. Other work demonstrated that GDNF (10 ng/ml) in human embryonic dopamine neurons cultures nearly doubled dopamine neuron survival and reduced the rate of apoptosis from 6% to 3%.

C. Gene Supplementation of the Porous Collagen Scaffold

1. Background

Many strategies have been explored for spinal cord repair procedures including: the injection of therapeutic molecules; the transplantation of cells; and gene therapy. However, none of these methods has yet been shown to have a long-term beneficial effect. In procedures delivering neurotrophic factors, a localized therapeutic action is limited by the short half-life of the proteins *in vivo* and their dissolution. The inability to maintain therapeutic levels for a prolonged period could be overcome by the use of a scaffold as a carrier for genes. The structural and material properties of the biomaterial scaffold are of importance for insuring a targeting action. For pDNA release, a porous structure with high surface area could provide a sustained, localized and high concentration of the plasmid. Furthermore, the degradation of the scaffold, controlled by the cross-linking density, could provide a prolonged release of a selected gene as the scaffold is replaced by the tissue ingrowth. A scaffold could also provide support for the migration of endogenous cells into the defect, and the migration of the transfected cells, seeded into the scaffold prior to implantation, from the defect site into surrounding tissue.

The objective of this part of the project was to develop a gene-supplemented collagen scaffold to have a long shelf life, an optimized release of plasmid-lipid complexes (lipoplexes) for the endogenous cells and the delivery of transfected cells to the defect over a period of several weeks when implanted.

Collagen scaffolds were made by a freeze-drying technique and prepared with various amounts of collagen, various cross-link densities, and with various freezing temperatures. The effect of gene supplementation on the cross-link density was evaluated using a swelling ratio. Finally, cell seeding and incorporation into the scaffolds were investigated for 2 different scaffold conditions, Pilot 1 and 2. Pilot #1: Scaffolds were made using a 1% collagen concentration with an annealing temperature of -40°C. Scaffolds were seeded with MSCs using different techniques and were evaluated after 24 hours. Pilot #2: Collagen scaffolds were made with various pore sizes and seeded with MSCs under static conditions, and evaluated after 1 week to determine the most promising scaffold for cell incorporation. For both experiments, DNA content of the scaffold was measured with a PicoGreen Assay, cell localization within the scaffold was visualized by histology and cell-mediated contraction was evaluated with the effective diameter of the scaffolds.

2. Materials and Methods

The porous collagen sheets were fabricated from type I/III, micro-fibrillar collagen derived from porcine (Geistlich Biomaterials, Wolhusen, Switzerland). Various concentrations of collagen were explored, ranging from 0.5% to 2% (w/w). Briefly, the collagen suspensions (pH=3) were prepared by mixing the collagen with a 0.001 N hydrochloric acid solution (Mallinckrodt Chemicals). This step induced the swelling of the collagen fibrils and the conversion of about 90% of banded collagen fibers to an unbanded structure. The slurries were first cooled at 0°C to prevent the denaturation of the collagen fibrils during blending and then mixed with a blender at 15,000 rpm for a total of 20 min. The final suspension was degassed by spinning at 1000 RCF (Relative Centrifugal Force) for 3 min. The collagen suspension was poured into plastic molds 15 cm x 3.7 cm and equilibrated to 20°C in the freeze-dryer for 15 min. Then the temperature of the freeze-dryer chamber (VirTis, Gardiner, NY) was decreased at a controlled rate of freezing to -40°C or -10°C. Previous studies have shown that the ramping process makes more homogeneous pores in the fabrication of the scaffolds. Once the final

temperature was reached, the temperature was annealed for a minimum period of 60 min. to allow the nuclei of ice crystals to form. After the solidification step, the suspension was converted to a dry foam through the process of lyophilization, by which the ice is removed by sublimation, leaving an empty pore in the place of every ice crystal. Hence, the lower the final temperature and the higher the rate of freezing, the smaller the final pore diameter. After this annealing period, the ice crystals were sublimated under vacuum (<200 mTorr) at 0°C for 17 hours to leave behind a homogeneous and highly porous solid collagen scaffold. Similar scaffolds have been previously reported in previous studies to have pore sizes of 151 μm for 0.5% collagen with an annealing temperature of -10C and 96 μm for 0.5% collagen with an annealing temperature of -40°C.

Dehydrothermal (DHT) treatment through drastic dehydration forms inter-chain peptide bonds between amino acid residues. DHT results in a decrease in the free amine groups and water-binding capacity, and it increases the tensile strength. Moreover, it has the effect of sterilizing the collagen scaffold. Immediately after the freeze-drying process, the porous sheets were cross-linked by DHT treatment under a vacuum of 50 mTorr and a temperature of 105°C for 17 hours.

Following the DHT treatment, 8-mm-diameter disks were cut from the porous sheets using a sharp-edged punch. Additional cross-linking was obtained by treatment with a water-soluble carbodiimide. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) is a water-soluble agent which catalyzes collagen cross-linking. Since it is not incorporated into the amide cross-links it can readily be washed out of the scaffold. EDAC treatment results in collagen-collagen bonds. Carboxylic groups of glutamic and aspartic acid residues are activated and form amide bonds in the presence of lysine or hydroxylysine residues. N-hydroxysuccinimide (NHS) increases the rate and amount of cross-linking. Prior work found little benefit is derived beyond 2 hours in the EDAC/NHS solution.

The 8-mm-diameter disks were immersed in a solution containing EDAC (Sigma Chemicals) and N-hydroxysuccinimide (NHS, Sigma) at various molar ratios of EDAC:NHS:carboxylic acid:

Treatment	EDAC:NHS:COOH
DHT	24hr, 105C, 30 mTorr
EDAC 1	1 :0.4 :1
EDAC 2	5 : 2 :1
EDAC 3	14 : 5.5 :1

The cross-link density for randomly coiled polymer networks is inversely related to the swelling ratio. Collagen scaffolds were gelatinized in water at 90°C for 2 min., placed between sheets of filter paper under a 1 kg weight for 20 sec. to expel excess water remaining in the pores, and then the scaffolds were weighed to determine the wet mass (M_W). Matrices were then dried in a oven at 110°C overnight and weighed to determine the dry mass (M_D). The swelling ratio, r*, was determined as follows:

$$\mathbf{r^* = 1/Vf = [(M_D/\rho c) + ((M_W-M_D)/ \rho water)] * \rho c/M_D}$$

r* = swelling ratio
Vf = volume fraction of dry collagen
ρc = density of collagen = 1.32 g/cm3
ρwater = density of water = 1 g/cm3

Solutions of the plasmid-lipid complex were pipetted into the collagen scaffolds according the plan shown in Fig. 7.

Two pilot studies for the cell seeding experiments were conducted. The first study (Pilot 1) aimed to optimize the incorporation of the rat MSCs after 24 hours in static and dynamic (orbital shaking) conditions. Scaffolds were made at a concentration of 1% collagen and had a subsequent EDAC treatment (EDAC 1 and EDAC 3). The cell density was 1 million cells per 8-mm-diameter disk. The objective of the second study (Pilot 2) was to evaluate the incorporation of the rat MSCs after 1 week of static seeding. Four different pores size were evaluated and the scaffolds were made at 0.5% and 1% collagen with T (annealing) = -10°C and -40°C and had a subsequent EDAC treatment (EDAC 1). The cell density was 500,000 cells per 8-mm disk.

Both pilot studies followed the same cell-seeding method (Fig. 8). At the day of seeding, the excess water in the hydrated scaffolds was quickly removed on sterile filter paper (Fisherbrand, Fisher Scientific Co) and the scaffolds were placed onto the 24 well-plates previously coated with 500ul of 3% agarose (m/w). The scaffolds were subsequently cross-linked with 1 ml of EDAC solution for 30 min. Then the scaffolds were rinsed for 30 min in PBS. After the cross-linking step, 10 µl of MSC suspension was pipetted onto one surface. After 10 min, the scaffolds were flipped over and an additional 10 µl of the cell suspension was added, and the cell-seeded scaffold left for 10 min at room temperature. Finally 1 ml of DMEM low glucose, 20% FBS and 1% antibiotic-antimycotic solution was added to each well.

The cell-seeded scaffolds were cultured in an incubator at 37°C in a 5% CO_2 atmosphere and 95% humidity. For long-term seeding, the medium was changed every 3 days.
The diameter of the scaffolds was measured with every change of medium. Cell-mediated contraction was calculated by dividing the average seeded effective diameter at a specific time point by the initial diameter of the matrix at Day 0. The cell-seeded scaffolds were terminated at 24 hours and 1 week after seeding, and the DNA content was determined with a PicoGreen Assay. Two disks per condition were terminated at the end of the experiment (after 24 hours for Pilot 1 and after 7 days for Pilot 2) for histology with hematoxylin and eosin (H&E) staining.

The aim of the following experiments was to evaluate the cell incorporation into the scaffolds. The experimental conditions are presented in the table below; the variables studied were the collagen concentration, annealing temperature and cross-link density of the scaffolds and the method of seeding (static or dynamic):

VARIABLES	Exp.#	Collagen Conc. (%)	T freezing (°C)	EDAC: NHS:COOH	Exp. Cond.	n
Cell incorporation in scaffolds with different seeding and cross-linking methods **(1 day)**	1-1	1%	-40	1:0.4:1	static	4
	1-2	1%	-40	14:5.5:1	static	4
	1-3	1%	-40	1:0.4:1	Rocking plate	4
	1-4	1%	-40	14:5.5:1	Rocking plate	4
	1-5	1%	-40	1:0.4:1	Orbital shaking	4
	1-6	1%	-40	14:5.5:1	Orbital shaking	4
Cell incorporation in scaffolds with different pore sizes **(1 week)**	2-1	0.5%	-10	1:0.4:1	static	8
	2-2	0.5%	-40	1:0.4:1	static	8
	2-3	1%	-10	1:0.4:1	static	8
	2-4	1%	-40	1:0.4:1	static	8

3. Results

The relative cross-link densities of the scaffolds were determined indirectly by means of the swelling ratio. The present study compared the effect of the amount of collagen (0.5% and 1%, w/w), the cross-link technique (DHT and three different concentrations of EDAC), and the presence of plasmid DNA, on the swelling ratio (Fig. 9). Three-factor ANOVA showed significance of collagen concentration ($P<0.0001$, power= 0.997), cross-linking density ($P<0.0001$, power=1) and gene incorporation ($P<0.0001$, power=1) on the inverse of swelling ratio. Further analysis with Fisher's PLSD test confirmed significance of the DNA load on the cross-linking density.

The swelling ratio of the collagen scaffolds, calculated as the inverse of the volume fraction of collagen ranged for scaffolds at 1% collagen varied from 5.0 ± 0.2 (mean ± standard deviation) for the DHT scaffolds to 3.6 ± 0.2 for the EDAC 3 scaffolds, and for scaffolds at 0.5% collagen from 5.0 ± 0.5 (mean ± standard deviation) for the DHT scaffolds to 2.7 ± 0.2 for the EDAC 3 scaffolds (Fig. 10). Taking the cross-link density to be proportional to the inverse of the swelling ratio, the density of the cross-links increased with the different cross-linking methods as follows: DHT< EDAC 1< EDAC 2< EDAC 3 (Fig. 10). Two-factor ANOVA showed significant effects of both the collagen concentration and the cross-linking treatment on the swelling ratio ($P<0.0001$; power=1). Further analysis with Fisher's PLSD demonstrated that the effect of EDAC 2 and 3 was not significantly different. Also, according to the results, the cross-link density increased as the amount of collagen in the scaffold decreased.

The swelling ratio of the collagen scaffolds calculated as the inverse of the volume fraction of collagen ranged for scaffolds at 1% collagen from 5.6 ± 0.4 (mean ± standard deviation) for the DHT scaffolds to 4.0 ± 0.2 for the EDAC 3 scaffolds and for scaffolds at 0.5% collagen from 5.9 ± 1.2 (mean ± standard deviation) for the DHT scaffolds to 3.8 ± 0.6 for the EDAC 3 scaffolds. The inverse of the swelling ratio followed the same trend as seen with the

unloaded collagen scaffolds but with a moderate impact: the density of the cross-links increased with the different cross-linking methods as follows: DHT< EDAC 1< EDAC 2< EDAC 3. Two-factor ANOVA showed that there were significant effect of the cross-linking treatment ($P<0.0001$; power=1) and no significant effects of the collagen concentration and on the swelling ratio (power=0.5).

The following table presents the main results obtained 24 hours and 1 week after cell seeding. DNA content, number of cells and relative number of cells were obtained after a PicoGreen Assay and the effective diameter was measured with a template.

Exp.#	DNA content (ng)	Number of cells	Final amount cells/initial (%)	Effective diameter (%)
1-1	7900±1700	790,000	79	NA
1-2	6400±2600	640,000	64	NA
1-3	5300±2100	530,000	53	NA
1-4	4300±2300	430,000	43	NA
1-5	7100±85	710,000	71	NA
1-6	4400±627	440,000	44	NA
2-1	1160±190	116,000	23	56±0.1
2-2	1660±290	166,000	33	92±0.1
2-3	2340±300	234,000	47	90
2-4	2330±320	233,000	47	100

The number of cells attached to the scaffolds after 1 day (n=2), based on the DNA contents, varied from about 40-70% of the number of cells seeded into the samples (*i.e.*, 1,000,000). The constructs cultured in static conditions contained about 790,000 cells/scaffold for EDAC 1 and 640,000 cells/scaffold for EDACS 3, whereas the scaffolds cultured in dynamic conditions contained about 530,000 cells/scaffold (EDAC 1) and 430,000 cells/scaffold (EDACS 3) for the rocking plate and about 710,000 cells/scaffold (EDAC 1) and 440,000 cells/scaffold (EDACS 3) for the orbital shaking. While the DNA analysis revealed more cells in the scaffolds cultured under the static condition, histology demonstrated a better incorporation of the MSCs into the scaffolds cultured under dynamic conditions.

After 2 weeks of culture, MSCs did not infiltrate significantly the gene-supplemented scaffolds at 1% collagen and $T_{Freezing}$=-40°C. Cells were mainly localized onto the surface of the scaffolds where they kept their rounded morphology. Some cells could be noted inside few very large pores. No contraction was observed after the 2-week experiment.

4. Summary

The results demonstrated how selected cross-linking treatments can be employed to control the cross-link density (swelling ratio) of collagen scaffolds. The results showed that an increased concentration of carbodiimide and a lower concentration of collagen provided a lower swelling ratio, hence a higher cross-link density. The fact that highest cross-link density could be observed for the scaffolds with the lowest collagen concentration may be related to a more open structure where possible sites for cross-links initiation would be more accessible.

The pilot seeding studies showed that the number of MSCs attached to the scaffold did not change significantly with the method of seeding and the cross-link density in the first 24-

hour period after seeding. However, histology demonstrated that the dynamic culture conditions, particularly orbital shaking, could help with the incorporation of the MSCs in the scaffolds.

Another important finding was the incorporation of the MSCs in scaffolds with different pore sizes. The results showed an increased number of cells attached to the scaffold with smaller pore size after 1 week. However, histology confirmed that only the scaffolds with the biggest pore size would actually allow the cells to reach the inner portions of the scaffold. It seems that the cells were able to migrate through the scaffolds with the biggest pore sizes.

The cell-mediated compression was effectively prevented during the two-week experiment in the scaffolds with the highest cross-link density.

In conclusion, this experimentation demonstrated the effect of the gene supplementation on the mechanical behavior (reflected in swelling ratio) of the collagen scaffolds, and the effect of the scaffold design on the cell incorporation.

D. Transfection of MSCs Grown in the Gene-Supplemented Porous Collagen Scaffold, with Plasmid DNA Encoding GDNF

1. Background

The objective of this aspect of the work was investigate the effect of the properties of a gene-supplemented collagen scaffold on the enhancement of the gene transfection of MSCs. The specific aim was to use a gene-supplemented collagen scaffold for the non-viral delivery of p-GDNF to MSCs. Type I/III porcine collagen scaffolds incorporated the gene encoding for GDNF. GP2 was selected as the transfection reagent for the experiments because it was shown in the monolayer studies to provide higher gene expression than Lipofectamine 2000.

The following studies evaluated different parameters to optimize the transfection in 3D. The profile of production of GDNF was studied for different cross-link density, and GDNF synthesis was evaluated relative to the initial plasmid doses (2 and 10 µg) and plasmid-transfection reagent ratio. Finally the effect of the pore size and static and dynamic culture methods were tested to optimize the *in vitro* conditions for the plasmid uptake and expression by the MSCs. The experimental matrix (below) for the optimization for the transfection in the gene-supplemented scaffolds was designed such that any experiment could be compared with a reference (group 1).

VARIABLES	Exp. # Group	Collagen conc. (%)	T Freezing (°C)	pDNA: GP2 (µl:µl)	EDAC: NHS: COOH	Exp. Cond.	n
Static vs dynamic	**1 (ref)**	0.5	-10	2:10	1:0.4:1	Static	8
	2	0.5	-10	2:10	1:0.4:1	Dynamic	8
Cross-link density	**3**	0.5	-10	2:10	14:5.5:1	Static	8
pDNA:GP2	**4**	0.5	-10	10:50	1:0.4:1	Static	8
	5	0.5	-10	2: 20	1:0.4:1	Static	8
Pore size	**6**	0.5	-40	2:10	1:0.4:1	Static	8
	7	1	-10	2:10	1:0.4:1	Static	8
	8	1	-40	2:10	1:0.4:1	Static	8

2. *Materials and Methods*

Excess of water in the hydrated collagen scaffolds, prepared as previously described, was removed on sterile filter paper and the scaffold placed onto 24-well-plates previously coated with agarose. The pGDNF-GP2 complex (20-25µl) was subsequently pipetted onto the surface of the scaffold and left at room temperature for 1 hour. After the complex was soaked into the scaffold, 1 ml of EDAC solution was added for 30 min. at room temperature. Then, the EDAC solution was aspirated and replaced by a solution of PBS. The scaffolds were rinsed for 30 min. After the cross-linking step, the scaffolds were flipped over and another 20-25 µl of pGDNF-GP2 solution was pipetted onto the surface of the scaffold and left at room temperature for 1 hour. The scaffolds were then ready for cell seeding.

The gene-supplemented scaffolds were seeded with rat MSCs (500,000 cells/scaffold), using the method previously described. Briefly, 10 µl of a suspension containing 250,000 cells in DMEM low glucose was pipetted onto the surface of the scaffolds. After 10 min. of incubation at room temperature, the scaffolds were flipped over and another 10 µl of a suspension containing 250,000 cells in DMEM low glucose was pipetted onto the surface. The scaffolds were left at room temperature for 10 min. Then, 0.5 ml of serum-free medium (DMEM low glucose) was added to the 24-well plates and incubated at 37°C. After 4 hours of transfection, 0.5 ml of MSCs medium (DMEM low glucose, 20% FBS, 1% antibiotic-antimycotic) was added. Finally another 0.5 ml of MSCs medium was added after 24 hours.

The media were collected (1 ml) on days 3, 6, 9, 12, and 14 post-transfection for ELISA analysis (Duo-Kit ELISA for the human GDNF protein (R&D Systems) and stored at -20°C until ELISA was performed. Chemiluminescent ELISA assay (R&D Systems) was run in a multilabel Victor 3 microplate counter using Wallac 1420 software (Perkin Elmer Life Sciences). The amount of GDNF released in the medium was then monitored by reading absorbance at 450 nm minus absorbance at 540 nm to correct optical aberrations.

The gene-supplemented scaffolds were terminated 2 weeks after seeding and the DNA content (reflecting the number of cells) was determined with a PicoGreen Assay (n=6).

Two discs per condition were allocated at the end of the experiment for histology staining.

3. *Results*

The following table presents the main results obtained after 2 weeks of 3D transfection.

Exp.# Group	Accumulated GDNF (ng/ml)	DNA content (ng)	Number of cells	Final amount cells/initial (%)	Scaffold contraction (%)
1	5.7±0.4	296±18	300E3±18E3	59	34±4
2	6.7±0.4	281±30	280E3±30E3	56	37±5
3	4.3±0.6	733±86	735E3±86E3	147	15±3
4	16±2.7	534±42	535E3±42E3	107	34±7
5	5.3±0.9	604±81	605E3±81E3	121	58±7
6	3.1±0.4	365±79	365E3±79E3	73	13±1
7	5.1±1.2	402±94	400E3±94E3	80	34±11
8	2.5±0.6	531±57	530E3±57E3	106	20±9

Cultures of MSC-seeded gene-supplemented grown under static (Group 1) and dynamic (Group 2) conditions demonstrated a monotonic increase in the accumulated GDNF over the 2-week time course of the experiment to approximately 5.5-6.5 ng/ml (Fig. 11). Group 2 samples, cultured under dynamic conditions, showed a higher GDNF production, and similar synthesis profile, compared to the static cultures. At the end of the 2-week period, the total of accumulated GDNF for the group in dynamic conditions was about 18% higher than the group cultured in static conditions. Two-factor ANOVA showed a significant effect of time in culture ($p<0.0001$, power= 0.997), and a significant effect of the transfection method ($p<0.002$), on the synthesis of GDNF by the rat MSCs.

DNA content at the end of the 2-week period of culture in dynamic conditions showed no noticeable difference in the results compared to the reference group. The gene-supplemented scaffolds cultured in static conditions contained about 300,000 cells/scaffold and the scaffolds cultured in dynamic conditions contained about 280,000 cells/scaffolds.

The cell-seeded scaffolds underwent a minimum of 35% reduction in diameter by the end of the 2-week period of culture. The contraction profiles for the scaffolds cultured in static and dynamic conditions were similar.

Group 1 samples, cross-linked with EDAC 1, showed a noticeable elevation in GDNF expression throughout the duration of the experiment compared to group 3 (Fig. 12 top). At the end of the 2-week period, the total accumulated for group 1 was about 33% higher than group 3. The profiles of production of GDNF were similar for both cross-link conditions, with a peak in GDNF synthesis on day 6 (Fig. 12 bottom). However, the concentration of GDNF found in the medium was much higher for group 1 with about 2.6 ng/ml than group 3 (1.6ng/ml), on day 6. Two-factor ANOVA showed a significant effect of culture time ($p<0.0001$, power= 0.997) and a significant effect of the cross-link density ($p=0.0071$, power=0.793) on the accumulated synthesis of GDNF by the rat MSCs. Further analysis with Fisher's PLSD post-hoc test on the temporal effect showed that there is 1) a significant effect of the cross-link density in the production of GDNF on day 6 ($p=0.46$) and 2) no significant difference in GDNF synthesis after 9 days.

After 2 weeks of culture in static conditions, the group with the highest cross-link density showed clearly higher values in DNA content compared to the reference group. The gene-supplemented scaffolds with the lowest cross-link density (EDAC 1) contained about 300,000 cells/scaffold whereas the scaffolds cross-linked with a higher dose of EDAC contained about 735,000 cells/scaffolds. The number of cells at the end of the experiment counted for, respectively, 59% and 147% of the initial amount of cells seeded into the scaffolds. One-factor ANOVA showed a significant effect of the cross-link density ($p<0.0005$, power= 0.997) on the number of cells in the scaffolds during the 3D transfection.

After a two-week experiment, the scaffolds with the lowest cross-link density (EDAC 1) had contracted about twofold more than the more heavily cross-linked scaffolds (EDAC 3). The higher stiffness of the higher cross-link density likely provided more resistance to the cell-mediated contraction.

Histology with H&E staining showed many more cells near the surface of the scaffolds than within. The MSCs seemed to have kept their rounded morphology inside the pores.

Group 4 specimens, with the highest plasmid load, showed a noticeable elevation in GDNF expression whereas the group with the highest ratio of plasmid to the transfection reagent (group 5) showed production of GDNF similar to the control group (Fig. 13). At the end of the 2-week period, the total accumulated of GDNF for the group 4 was about 180% higher than the

group 1 whereas for group 4, it was about 7% lower than the control group. The profiles of GDNF production were similar for the three conditions, with a peak at day 6 (Fig. 13 bottom). On day 6, the amount of GDNF released bys group 4 samples was more than 3-fold higher than the GDNF produced in the two other groups. However, the rate of GDNF synthesis decreased after day 9, and was not significantly different for all three groups after day 12. Two-factor ANOVA showed a significant effect of both the duration of the experiment ($p<0.0001$, power= 1) and the dose of plamid:transfection reagent ($p<0.0001$, power= 1) on the synthesis of GDNF by the rat MSCs. Further analysis with Fisher's PLSD test on the temporal effect showed that, after two weeks, there was a prominent effect of the amount of plasmid in the lipoplexes ($p<0.0001$) but no significant effect of the ratio plasmid load: transfection reagent ($p=0.68$) in the production of GDNF.

After 2 weeks of culture, both groups with the highest plasmid load and highest ratio plasmid to transfection reagent showed higher values in DNA content than the reference group. The gene-supplemented scaffolds cross-linked with the lowest load of plasmid and ratio to the transfection reagent (2 μg pGDNF: 10ul GenePorter2) contained about 300,000 cells/scaffold, whereas the scaffolds with a highest amount of plasmid (10 μg pGDNF: 50 μl GenePorter2) and a highest ratio to the transfection reagent (2 μg pGDNF: 20 μl GenePorter2) contained about 535,000 and 605,000 cells/scaffolds, respectively. The number of cells at the end of the experiment accounted for 60%, 105 and 120%of the initial amount of cells incorporated into the scaffold, respectively. One-factor ANOVA showed a significant effect of the dose of plasmid and transfection reagent ($p<0.0001$, power= 1) on the number of rat MSCs in the scaffolds during the 3D transfection.

After two weeks of culture, the scaffolds with the highest ratio of plasmid to transfection reagent had been contracted about two-fold more than the two others groups. These results need to be regarded relative to the number of cells found in the scaffolds. On day 14, the contraction normalized to the number of cells showed similar results for both 2 μg:10 μl (μg pGDNF:μl GP2) and 2 μg:20 μl conditions, whereas, the condition with the highest plasmid load demonstrated lower results.

H&E staining showed an important infiltration of the cells into the scaffold for all conditions after 2 weeks of experiment. MSCs in the pores of the scaffolds supplemented with 10 μg pGDNF: 50 μl GP2 appeared to have a morphology quite different from the expected rounded one. MSCs on the surface of the scaffolds previously supplemented with 2 μg pGDNF: 20 μl GP2 displayed an elongated morphology, with extracellular matrix evident within the pores and between the cells.

Group 1, with the biggest pore size (group 1) showed a prominent elevation in GDNF expression compared to the groups with smaller pore sizes (group 7 and 8) (Fig. 14). At the end of the 2-week period, the total accumulated GDNF for the group 6 was about 11% lower, group 7 about 46% lower and group 8 about 56% lower than the samples in group 1. The profiles of production of GDNF per collection period were different for all four groups (Fig. 14 bottom). The peak in GDNF synthesis was visible on day 6 for group 1, on day 3 for group 6 and on day 9 for group 7. The level of GDNF production was maintained constant for about 9 days for group 8. Two-factor ANOVA showed a significant effect of both the time in culture ($p<0.0001$, power= 1) and the scaffold design ($p<0.0001$, power= 1) on the synthesis of GDNF by the rat MSCs. Further analysis with Fisher's PLSD test showed that, after two weeks, there was no significant effect of the pore size between groups 1 and 6 ($p=0.59$) and between groups 7 and 8 ($p=0.56$).

After 2 weeks of culture, there was a pronounced trend in the DNA content per scaffold as the pore size changed. As the size of the pores decreased, the DNA content in the gene-supplemented increased. The implants with the smallest pore size (1% collagen scaffolds with $T_{freezing}$=-40°C) contained about 530,000 cells/scaffold, whereas the scaffolds with a bigger pore size (0.5% collagen scaffold with $T_{freezing}$=-10°C) contained about 300 000 cells/scaffolds. The intermediate pore sizes (0.5% collagen scaffolds with $T_{freezing}$=-40°C; 1% collagen scaffolds with $T_{freezing}$=-10°C) contained about 365 000 and 400 000 cells/scaffolds. The number of cells at the end of the experiment accounted for, going from the biggest to the smallest pore sizes, 60%, 73%, 80% and 105% of the initial amount of cells seeded into the scaffold. One-factor ANOVA showed no significant effect of the pore size of the scaffold ($p<0.14$, power= 0.44) on the number of cells in the scaffolds during the 3D transfection. A post-hoc test, showed a significant difference in the incorporation and survival of the cells into the scaffolds ($p<0.025$), comparing the 0.5% collagen scaffold with $T_{freezing}$=-10°C and the 1% collagen scaffolds with $T_{freezing}$=-40°C.

Scaffolds with 0.5% collagen/ $T_{freezing}$= -10°C and 1% collagen/ $T_{freezing}$= -10°C had the highest contraction and a similar profile. Further analysis with a post-hoc test showed that after two weeks, there was no significant difference between the following conditions: 0.5% -10C (group 1) vs1% -10C (group 7) and 0.5% and -40°C (group 6) vs 1% and -40°C (group 8). Two-factor ANOVA showed a significant effect of the culture time ($p<0.0001$, power= 1) and the pore size ($p<0.0001$, power= 1) on the contraction of the scaffolds during the 3D transfection.

4. Summary

These results demonstrated that our collagen scaffold can be used as a delivery vehicle for the plasmid encoding for GDNF for the treatment of spinal cord defects. Selected parameters of the scaffolds were altered during the fabrication process (collagen concentration and freezing temperature) and the cross-linking treatment to study the effect of the pore size and the cross-linking density on the 3D transfection. The cross-linking treatment did not affect significantly the release profile of GDNF which could be explained by a mechanical entrapment of the lipoplexes in the collagen fibrils and not a direct cross-linking of the lipoplexes to the scaffold. The application of orbital shaking during the 4 hours of transfection had a slight positive effect on the production of GDNF, but not as strong as previously reported in monolayers studies.

The load of plasmid DNA, as expected, was a prominent parameter in the 3D transfection. The highest level of GDNF expression was observed for a dose of plasmid DNA and transfection time to be 10 µg and 6 days after transfection.

In conclusion, our work has demonstrated that gene-supplemented collagen scaffolds can serve as a carrier for lipoplexes and modified MSCs and provide a long-term overexpression of GDNF.

Table 1
Experimental Groups

Four experimental groups of matrices have been obtained by changing the collagen concentration and the freezing temperature. (*) The collagen suspension in Group I and II has been frozen by directly placing the copper-fitted end of the plastic mold onto the shelf of the freeze-dryer, pre-cooled at -40°C; for Group III and IV, the freezing has been achieved by placing the mold onto a copper plate, previously cooled with liquid nitrogen (LN_2).

	Collagen concentration % w/v	**Freezing temperature**
Group I	2%	-40°C (*)
Group II	0.5%	-40°C
Group III	2%	LN_2 (*)
Group IV	0.5%	LN_2

Table 2
Pore Diameter and Aspect Ratio in Cross-Sections of the Cylindrical Scaffolds

Pore size and pore aspect ratio, measured in the peripheral (outer 400 µm) and central (inner 1.2 mm of the scaffold diameter) areas of the scaffolds. Result are expressed as means ± the standard deviation of the mean (n=6). Both collagen concentration and freezing temperature affect significantly the mean pore size ($p<0.0001$).

	Central area		**Peripheral area**	
	Pore size (µm)	**Aspect ratio**	**Pore size (µm)**	**Aspect ratio**
Group I 2%, -40°C	40 ± 1	1.15 ± 0.08	52 ± 15	1.11 ± 0.11
Group II 0.5%, -40°C	62 ± 8	1.11 ± 0.04	55 ± 16	1.10 ± 0.10
Group III 2%, LN_2	23 ± 2	1.06 ± 0.08	32 ± 6	1.11 ± 0.09
Group IV 0.5%, LN_2	33 ± 5	1.11 ± 0.09	30 ± 5	1.11 ± 0.09

Table 3
Pore Orientation

Pore orientation expressed in terms of OI (mean ± the standard deviation of the mean; n=6). β is the angle formed between the axis of the pore channels and the longitudinal axis of the cylindrical scaffold. The orientation in the central area is nearly axial for all the experimental groups; the peripheral area is slightly more radially oriented compared to the central one.

	Central area		**Peripheral area**	
	OI	**Angle °**	**OI**	**Angle °**
Group I 2%, -40°C	0.92 ± 0.07	10.2 ± 4.8	0.80 ± 0.09	16.3 ± 4.6
Group II 0.5%, -40°C	0.89 ± 0.11	11.4 ± 6.4	0.80 ± 0.19	13.8 ± 8.6
Group III 2%, LN_2	0.77 ± 0.37	15.2 ± 13.3	0.55 ± 0.37	26.4 ± 13.5
Group IV 0.5%, LN_2	0.94 ± 0.05	8.9 ± 3.8	0.89 ± 0.11	9.6 ± 6.7

Table 4
Pore Volume Fraction and Surface Area

Values of pore volume fraction and specific surface area, calculated according to eq. (4) and (5) respectively. The value of specific surface area is referred to the mean pore diameter measured for the central area of the four experimental groups.

	Pore volume fraction %	**Specific surface area (mm^2/mm^3)**
Group I 2%, -40°C	98.4	200
Group II 0.5%, -40°C	99.5	133
Group III 2%, LN_2	98.4	400
Group IV 0.5%, LN_2	99.5	250

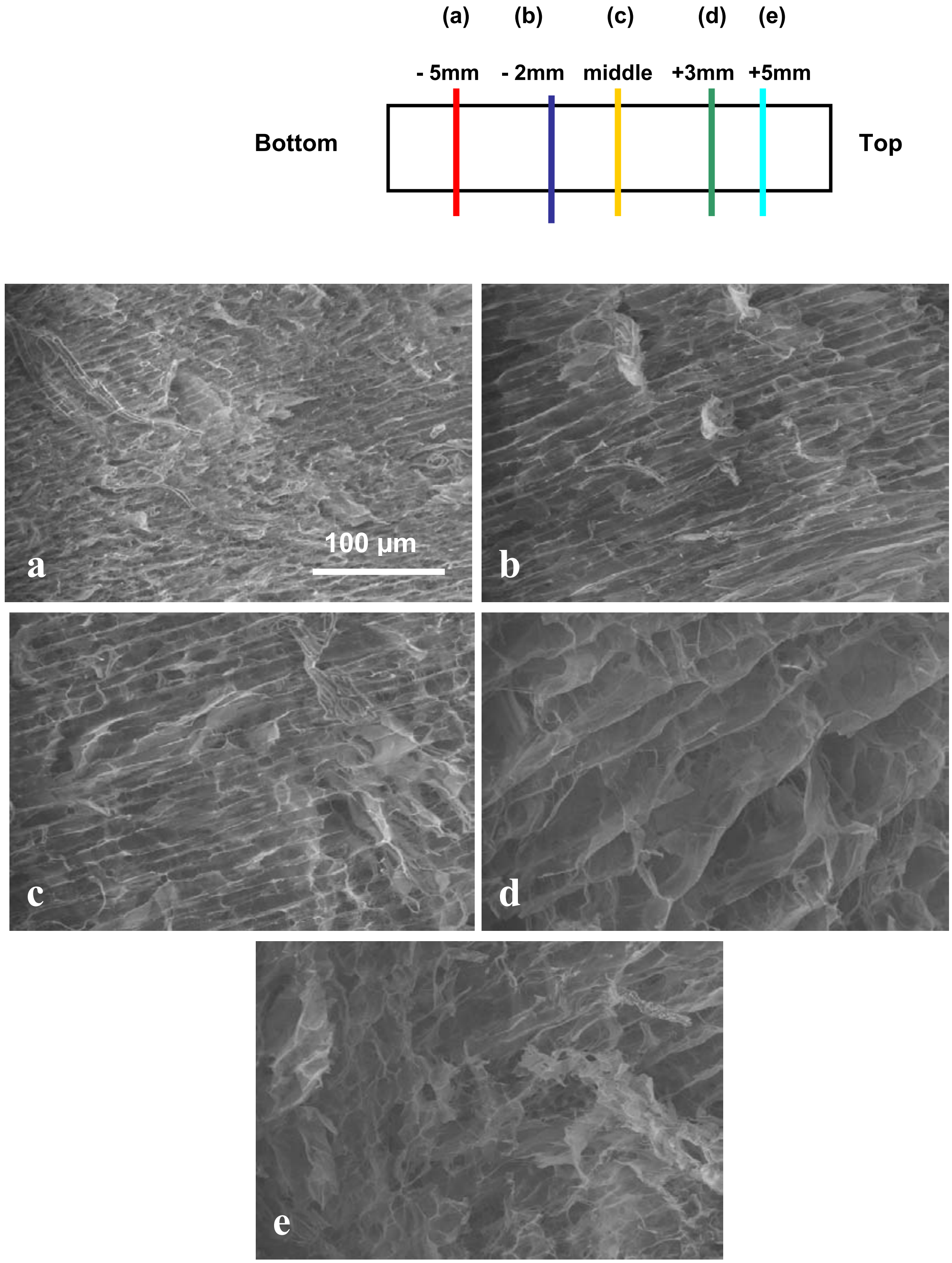


Fig. 1 Scanning electron microscopy images of the collagen scaffolds.

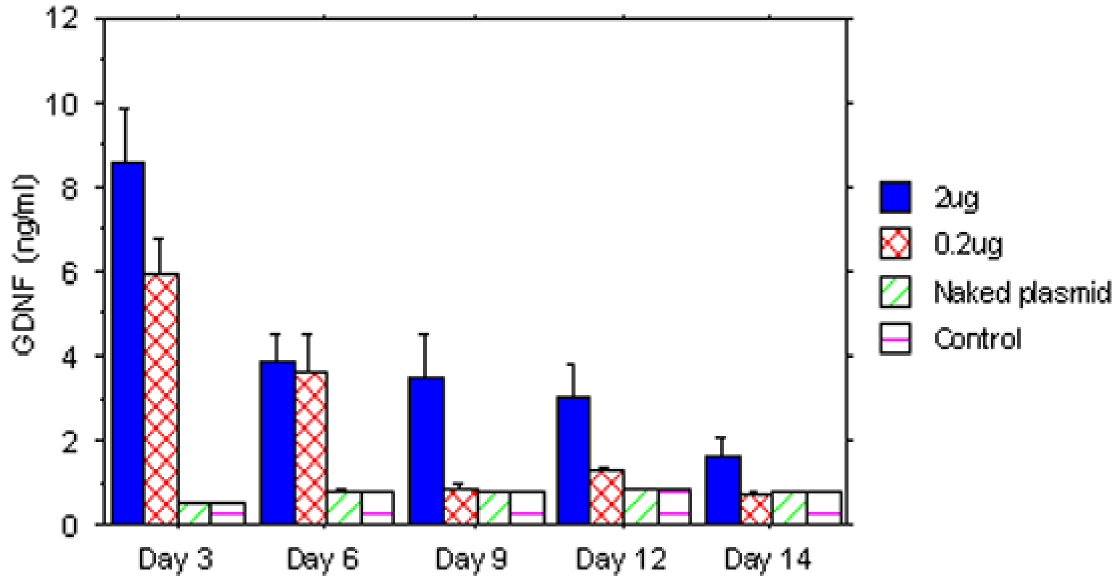


Fig. 2.: Release of GDNF (ng/ml) collected in the medium after transfection of rat MSC monolayers and using different loads of plasmid in the lipoplexes. n=6; mean±SEM.

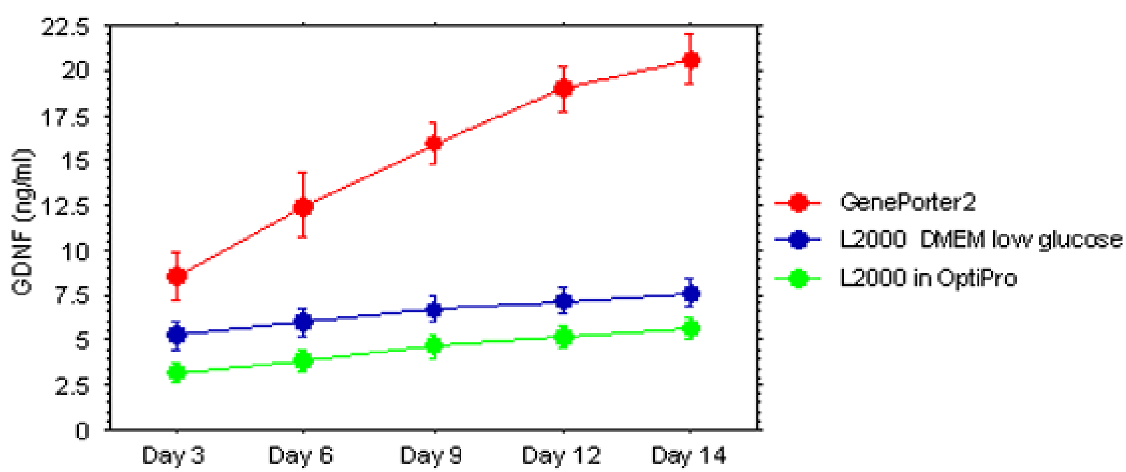


Fig. 3 Accumulated release of GDNF (ng/ml) into the medium after transfection of rat MSCs, using different transfection reagents. n=6; mean±SEM

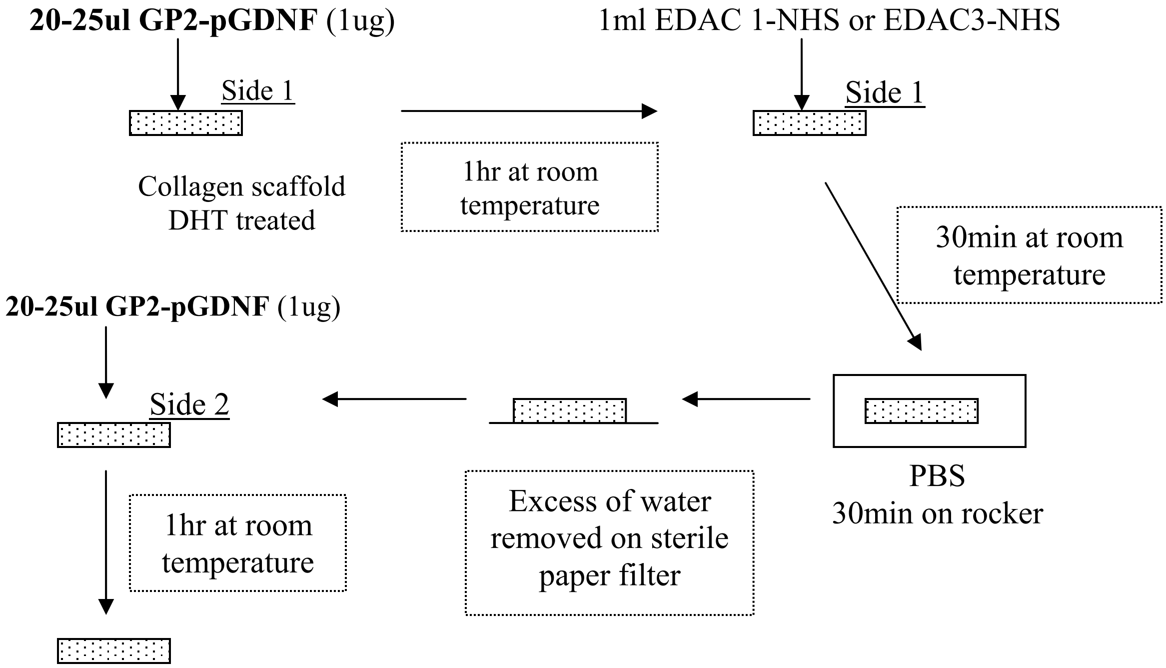


Fig. 4 Method of incorporation of the lipoplexes in the collagen scaffolds.

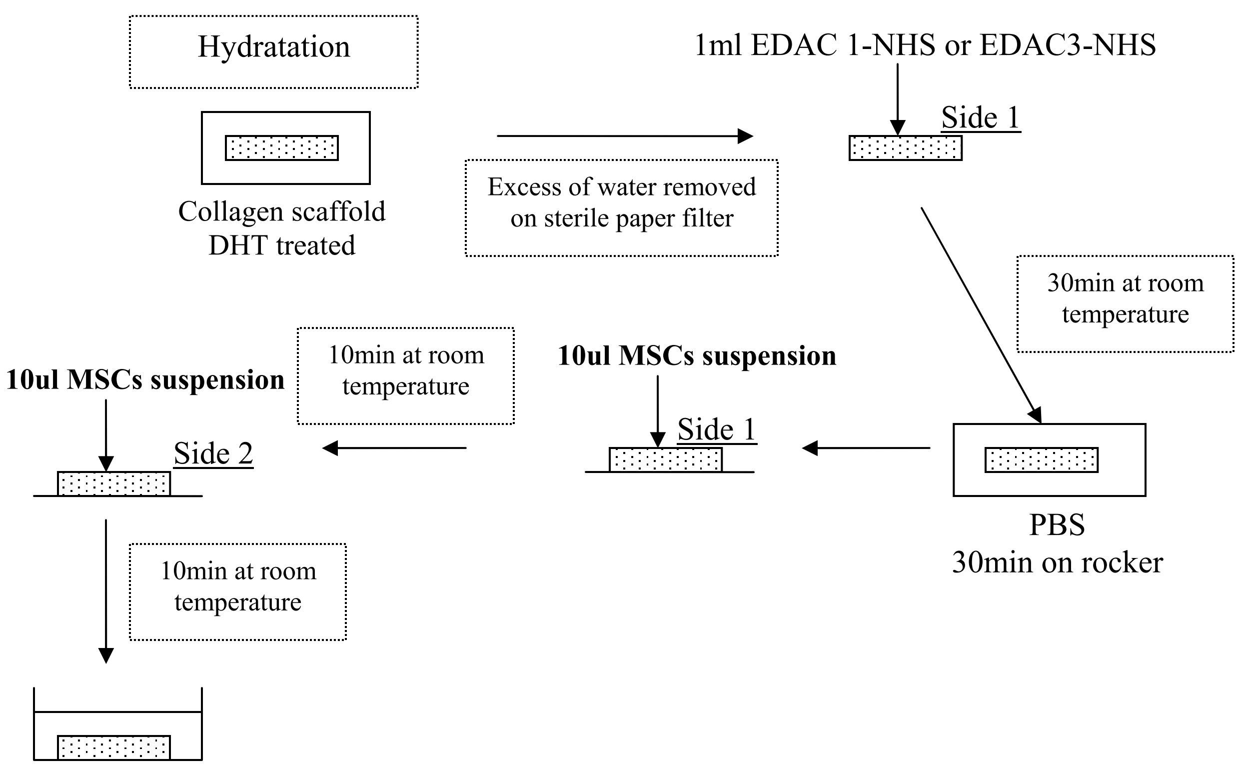


Fig. 5. Method of incorporation of the MSC suspension in the collagen scaffolds.

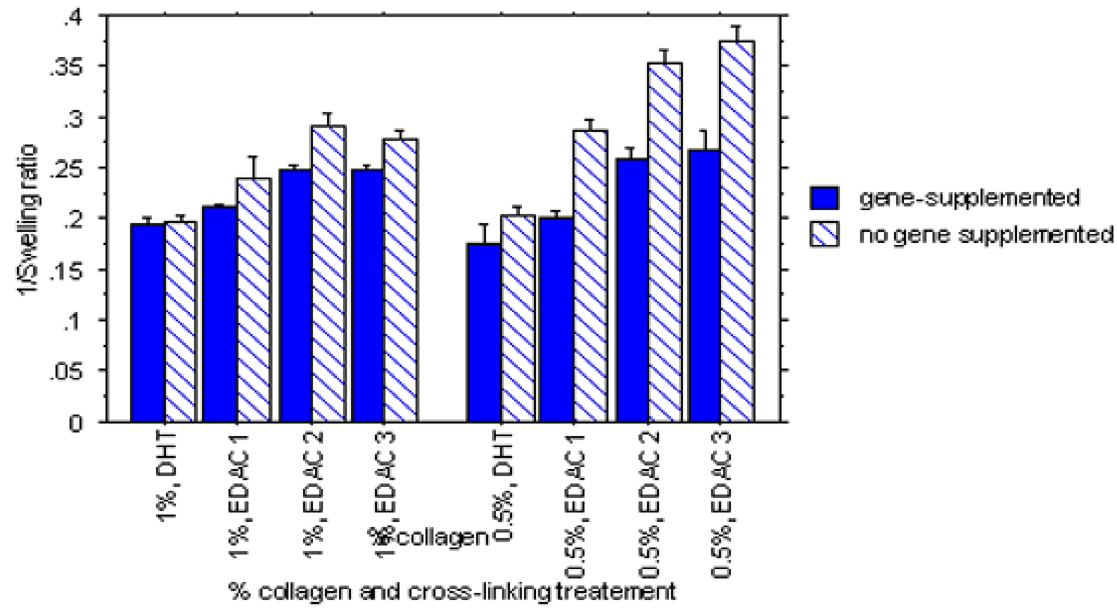


Fig. 6. Inverse of the swelling ratio for gene-supplemented scaffolds and control for various collagen concentration and cross-linking treatments. Mean±SEM; n=5.

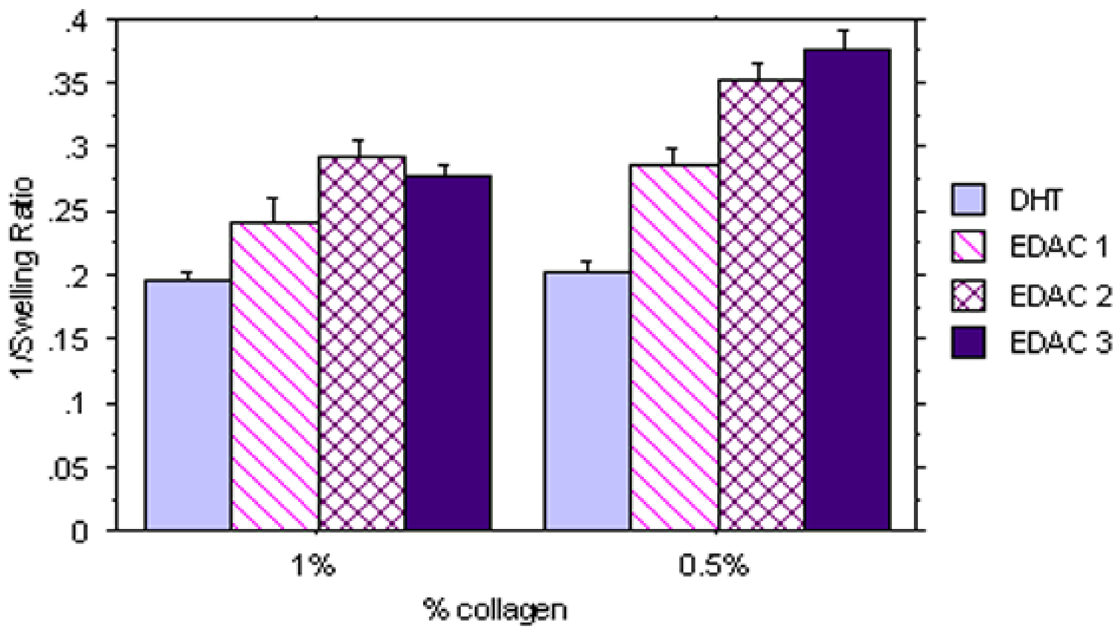


Fig. 7. Inverse swelling ratio of cross-linked scaffolds with different concentrations of collagen. Mean±SEM; n=5.

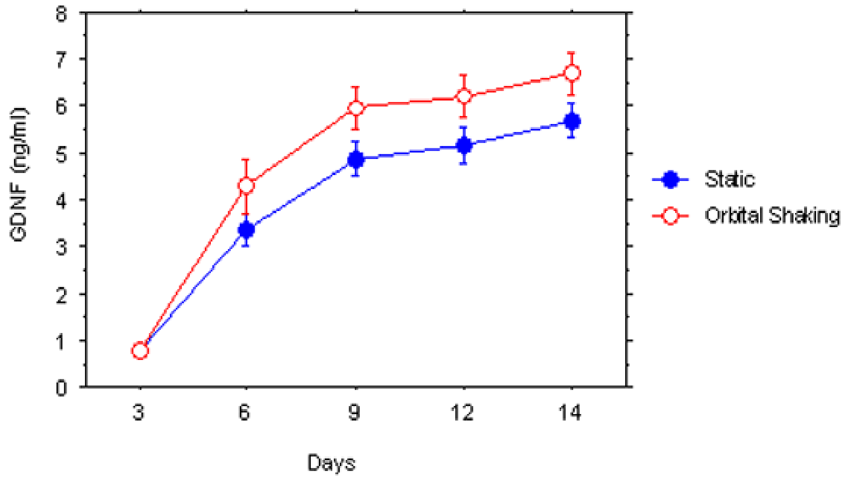


Fig. 8. Accumulated GDNF (ng/ml) released in the medium versus culture time for two different transfection methods (dynamic and static). The gene-supplemented scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=8.

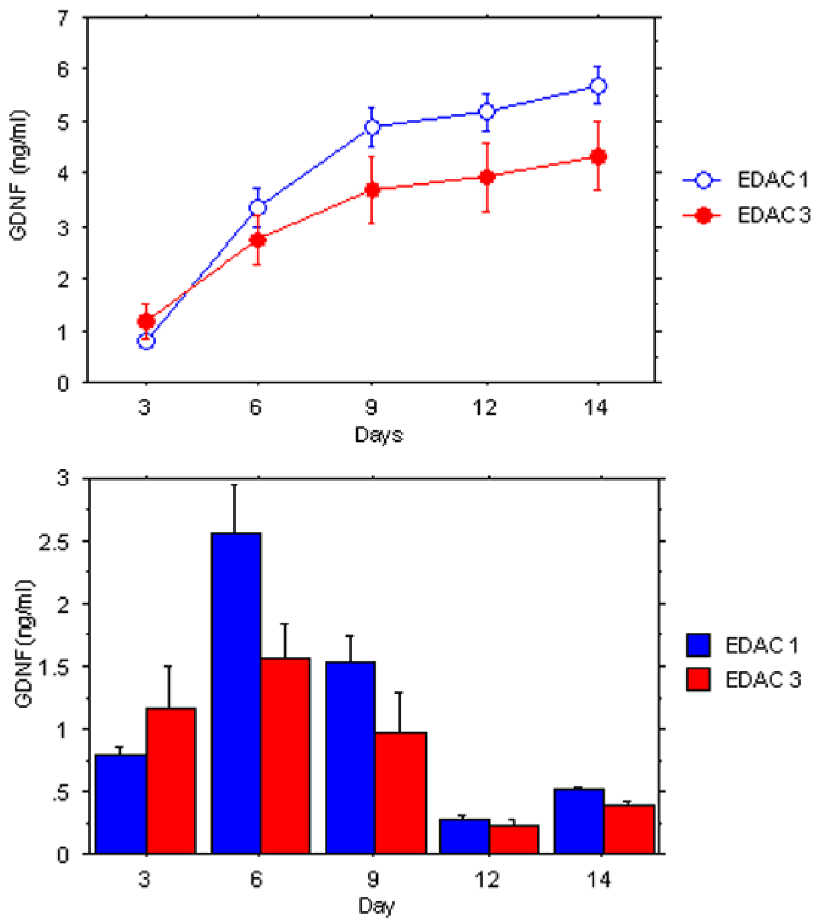
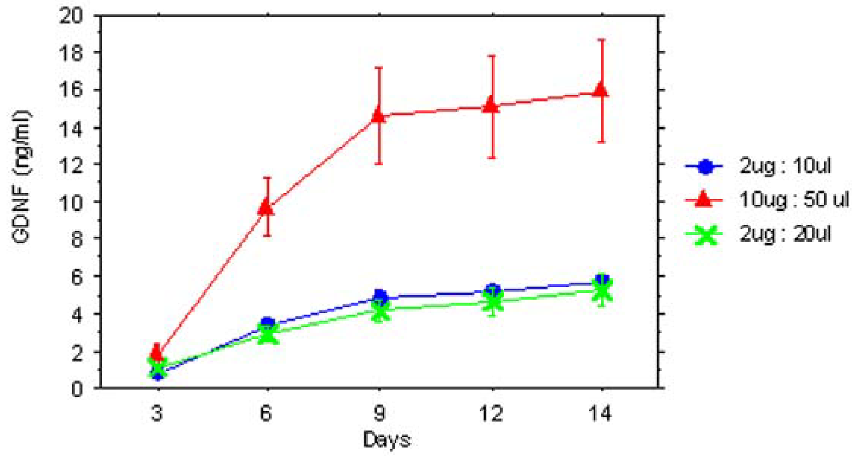


Fig. 9. Accumulated GDNF (ng/ml, top) and GDNF produced at the medium collection periods (ng/ml, bottom) versus culture time for two different cross-link densities (EDAC 1= 1:0.4:1 and EDAC 3= 14: 5.5: 1). The gene-supplemented scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=8.



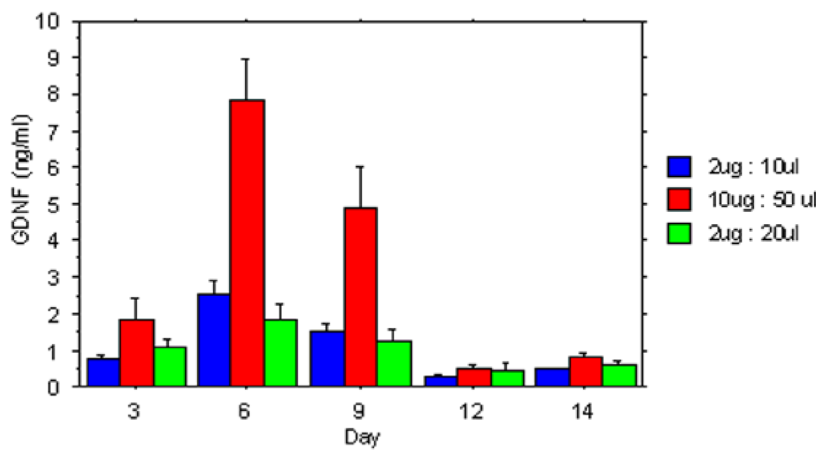
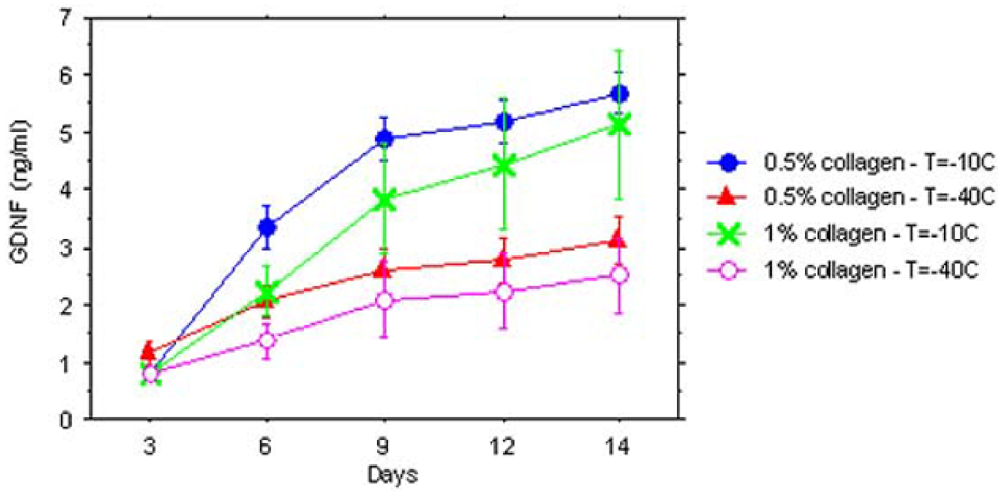


Fig. 10. Accumulated GDNF (ng/ml, top) and GDNF produced per collection period (ng/ml, bottom) versus culture time for three different doses of lipoplexes (pGDNF (µg): GenePorter2 (µl)). The gene-supplemented scaffolds were seeded with 500,000 rat mesemchymal MSCs per scaffold. Mean ± SEM; n=8.



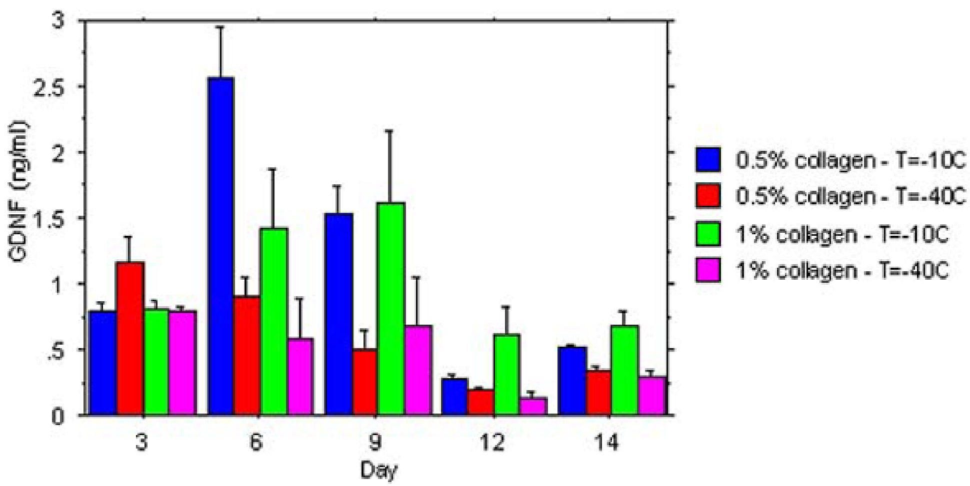


Fig. 11. Accumulated GDNF (ng/ml, top) and GDNF produced per collection period (ng/ml, bottom) versus culture time for four different pore sizes. The gene-supplemented scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=8.

III. KEY RESEARCH ACCOMPLISHMENTS

- Porcine type I/III collagen scaffolds were fabricated with uniaxially aligned pores.
- Conditions were found for the *ex vivo* non-viral transfection of marrow-derived mesenchymal stem cells with the plasmid DNA encoding an important neurotrophic factor, GDNF, in monolayer culture.
- Methods were determined to supplement collagen scaffolds with the GDNF plasmid.

IV. REPORTABLE OUTCOMES

1. Porcine type I/III collagen scaffolds were fabricated with oriented pores.
2. Rat marrow-derived mesenchymal stem cells were transfected with GDNF plasmid in monolayer culture.
3. Rat marrow-derived mesenchymal stem cells grown in collagen scaffolds incorporating the GDNF plasmid with displayed overexpression of GDNF *in vitro*.

V. CONCLUSIONS

These results demonstrated that our collagen scaffolds can be made with uniaxially oriented pores and can be used as a delivery vehicle for the plasmid encoding for GDNF for the treatment of spinal cord defects.

VI. REFERENCES

None

VII. APPENDICES

None